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<b>(21) International Application Number:</b> PCT/US94/14061 <b>(22) International Filing Date:</b> 7 December 1994 (07.12.94)  <b>(30) Priority Data:</b> 08/164,031 8 December 1993 (08.12.93) US  <b>(71) Applicant:</b> PROTOTEK, INC. [US/US]; 6497 Sierra Lane, Dublin, CA 94568 (US).  <b>(72) Inventors:</b> ZIMMERMAN, Mary, P.; 3727 Crofter's Court, Pleasanton, CA 94588 (US). SMITH, Robert, E.; 574 Escondido Circle, Livermore, CA 94550 (US).  <b>(74) Agents:</b> McNETT, John, C. et al.; Woodard, Emhardt, Naughton, Moriarty & McNett, Bank One Center/Tower, Suite 3700, 111 Monument Circle, Indianapolis, IN 46204 (US).		<b>(81) Designated States:</b> AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  <b>Published</b> <i>With international search report</i> <i>With amended claims.</i>
<b>(54) Title:</b> CYSTEINE PROTEASE INHIBITORS CONTAINING HETEROCYCLIC LEAVING GROUPS  <b>(57) Abstract</b>  A class of cysteine protease inhibitors which inactivate a cysteine protease by covalently bonding to the protease and releasing a heterocyclic leaving group is presented. The cysteine protease inhibitors of the present invention comprise a first portion which targets a desired cysteine protease and positions the inhibitor near the thiolate anion portion of the active site of the protease, and a second portion which covalently bonds to the cysteine protease and irreversibly deactivates that protease by providing a carbonyl or carbonyl-equivalent which is attacked by the thiolate anion of the active site of the cysteine protease to sequentially cleave a heterocyclic leaving group. The heterocyclic leaving group of the protease inhibitor is of the formula: - O - Het, where Het is a heterocycle having 4-7 atoms in the ring, with at least one of the heterocycle atoms being N, O or S.		

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CYSTEINE PROTEASE INHIBITORS  
CONTAINING HETEROCYCLIC LEAVING GROUPS

FIELD OF THE INVENTION

The present invention relates generally to cysteine  
5 protease inhibitors, and more particularly to cysteine  
protease inhibitors which are peptidyl ketones having  
heterocyclic leaving groups. The cysteine protease  
inhibitors of the present invention are particularly designed  
for the in vivo management of cysteine proteases,  
10 particularly cathepsins B, L, H and C, and their primitive  
enzymatic counterparts.

BACKGROUND TO THE INVENTION

Cysteine proteases associated with human disease states  
can be grouped into three categories: (1) lysosomal  
15 cathepsins; (2) cytosolic calpains; and (3) procaryotic  
enzymes with autocatalytic activation. Cathepsins B, H, and  
L are cysteinyl proteases involved in normal protein  
degradation. As such, they are generally located in the  
lysosomes of cells. When these enzymes are found  
20 extralysosomally they have been implicated by use of synthetic  
substrate technology and by natural endogenous inhibitors as  
playing a causative role in a number of disease states such  
as rheumatoid arthritis, osteo arthritis, pneumocystis  
carinii, schistosomiasis, trypanosoma cruzi, trypanosoma  
25 brucei brucei, Crithidia fusiculata, malaria, periodontal  
disease, tumor metastasis, metachromatic leukodystrophy,  
muscular dystrophy, etc. For example, a connection between  
cathepsin B-type enzymes and rheumatoid arthritis has been

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suggested in van Noorden and Everts, "Selective Inhibition of Cysteine Proteinases by Z-Phe-Ala-CH<sub>2</sub>F Suppresses Digestion of Collagen by Fibroblasts and Osteoclasts," 178 Biochemical and Biophysical Research Communications 178; Rifkin,

5 Vernillo, Kleekner, Auszmann, Rosenberg and Zimmerman, "Cathepsin B and L Activities in Isolated Osteoclasts," 179 Biochemical and Biophysical Research Communications 63; Grinde, "The Thiol Proteinase Inhibitors, Z-Phe-Phe-CHN<sub>2</sub> and Z-Phe-Ala-CHN<sub>2</sub>, Inhibit Lysosomal Protein Degradation

10 in Isolated Rat Hepatocytes," 757 Biochimica et Biophysica Acta 15; Mason, Bartholomew and Hardwick, "The Use of Benzyloxycarbonyl[<sup>125</sup>I]iodotyrosylalanyldiazomethane as a Probe for Active Cysteine Proteinases in Human Tissues," 263 Biochem. J. 945; van Noorden, Smith and Rasnick, "Cysteine

15 Proteinase Activity in Arthritic Rat Knee Joints and the Effects of a Selective Systemic Inhibitor, Z-Phe-Ala-CH<sub>2</sub>F," 15 J. Rheumatol. 1525; and van Noorden, Vogels and Smith, "Localization and Cytophotometric Analysis of Cathepsin B Activity in Unfixed and Undecalcified Cryostat Sections of

20 Whole Rat Knee Joints," 37 J. Histochemistry and Cytochemistry 617. A connection between cathepsin B and osteo arthritis has been suggested in Delaissé, Eeckhout and Vaes, "In Vivo and In Vitro Evidence for the Involvement of Cysteine Proteinases in Bone Resorption," 125 Biochemical and

25 Biophysical Research Communications 441; a connection between cathepsin B and pneumocystis carinii has been suggested in Hayes, Stubberfield, McBride and Wilson, "Alterations in Cysteine Proteinase Content of Rat Lung Associated with Development of Pneumocystis Carinii Infection," 59 Infection

30 and Immunity 3581; a connection between cysteine proteinases and schistosomiasis has been suggested in Cohen, Gregoret, Amiri, Aldape, Railey and McKerrow, "Arresting Tissue Invasion of a Parasite by Protease Inhibitors Chosen With the Aid of Computer Modeling," 30 Biochemistry 11221. A

35 connection between cysteine proteinases and trypanosoma

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- cruzi, trypanosoma brucei brucei and crithidia fasciculata has been suggested in Ashall, Harris, Roberts, Healy and Shaw, "Substrate Specificity and Inhibitor Sensitivity of a Trypanosomatid Alkaline Peptidase," 1035 Biochimica et
- 5 Biophysica Acta 293, and/or in Ashall, Angliker and Shaw, "Lysis of Trypanosomes by Peptidyl Fluoromethyl Ketones," 170 Biochemical and Biophysical Research Communications 923. A connection between cysteine proteinases and malaria has been suggested in Rosenthal, Wollish, Palmer and Rasnick,
- 10 "Antimalarial Effects of Peptide Inhibitors of a Plasmodium Falciparum Cysteine Proteinase," 88 J. Clin. Invest. 1467, and in Rosenthal, Lee and Smith, "Inhibition of a Plasmodium Vinckei Cysteine Proteinase Cures Murine Malaria," (in press). A connection between cathepsin B and tumor
- 15 metathesis has been suggested in Smith, Rasnick, Burdick, Cho, Rose and Vahratian, "Visualization of Time-Dependent Inactivation of Human Tumor Cathepsin B Isozymes by a Peptidyl Fluoromethyl Ketone Using a Fluorescent Print Technique," 8 Anti-cancer Research 525. A connection between
- 20 cathepsin B and cancer has been suggested in Gordon and Mourad, 2 Blood Coagulation and Fibrinolysis 735. A connection between cathepsin B and periodontal disease has been suggested in Cox, Cho, Eley and Smith, "A Simple, Combined Fluorogenic and Chromogenic Method for the Assay of
- 25 Proteases in Gingival Crevicular Fluid," 25 J. Periodont. Res. 164; Uitto, Larjava, Heino and Sorsa, "A Protease of Bacteroides Gingivalis Degrades Cell Surface and Matrix Glycoproteins of Cultured Gingival Fibroblasts and Induces Secretion of Collagenase and Plasminogen Activator," 57
- 30 Infection and Immunity 213; Kunitatsu, Yamamoto, Ichimaru, Kato and Kato, "Cathepsins B, H and L Activities in Gingival Crevicular Fluid From Chronic Adult Periodontitis Patients and Experimental Gingivitis Subjects," 25 J Periodont Res 69; Beighton, Radford and Naylor, "Protease Activity in Gingival
- 35 Crevicular Fluid From Discrete Periodontal Sites in Humans

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With Periodontitis or Gingivitis"; 35 Archs oral Biol. 329; Cox and Eley, "Preliminary Studies on Cysteine and Serine Proteinase Activities in Inflamed Human Gingiva Using Different 7-Amino-4-Trifluoromethyl Coumarin Substrates and  
5 Protease Inhibitors," 32 Archs oral Biol. 599; and Eisenhauer, Hutchinson, Javed and McDonald, "Identification of a Cathepsin B-Like Protease in the Crevicular Fluid of Gingivitis Patients," 62 J Dent Res 917. A connection between cathepsin B and metachromatic leukodystrophy has been  
10 suggested in von Figura, Steckel, Conary, Hasilik and Shaw, "Heterogeneity in Late-Onset Metachromatic Leukodystrophy. Effect of Inhibitors of Cysteine Proteinases," 39 Am J Hum Genet. 371; a connection between cathepsin B and muscular leukodystrophy has been suggested in Valentine, Winand,  
15 Pradhan, Moise, de Lahunta, Kornegay and Cooper, "Canine X-Linked Muscular Dystrophy as an Animal Model of Duchenne Muscular Dystrophy: A Review," 42 Am J Hum Genet 352; a connection between cathepsin B and rhinovirus has been suggested in Knott, Orr, Montgomery, Sullivan and Weston,  
20 "The Expression and Purification of Human Rhinovirus Protease 3C," 182 Eur. J. Biochem. 547; a connection between cathepsin B and kidney disease has been suggested in Baricos, O'Connor, Cortez, Wu and Shah, "The Cysteine Proteinase Inhibitor, E-64, Reduces Proteinuria in an Experimental Model of  
25 Glomerulonephritis," 155 Biochemical and Biophysical Research Communications 1318; and a connection between cathepsin B and multiple sclerosis has been suggested in Dahlman, Rutschmann, Kuehn and Reinauer, "Activation of the Multicatalytic Proteinase from Rat Skeletal Muscle by Fatty Acids or Sodium  
30 Dodecyl Sulphate," 228 Biochem. J. 171.

Connections between certain disease states and cathepsins H and C have also been established. For example, cathepsin H has been directly linked to the causative agents of Pneumocystis carinii and in the neuromuscular diseases  
35 Duchenne dystrophy, polymyositis, and neurogenic disorders.

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Stauber, Riggs and Schochet, "Fluorescent Protease Histochemistry in Neuromuscular Disease," Neurology 194 (Suppl. 1) March 1984; Stauber, Schochet, Riggs, Gutmann and Crosby, "Nemaline Rod Myopathy: Evidence for a Protease Deficiency," Neurology 34 (Suppl. 1) March 1984. Similarly, cathepsin C has been directly linked to muscular diseases such as nemaline myopathy, to viral infections, and to processing and activation of bone marrow serine proteases (elastase and granzyme A). McGuire, Lipsky and Thiele, "Generation of Active Myeloid and Lymphoid Granule Serine Proteases Requires Processing by the Granule Thiol Protease Dipeptidyl Peptidase I, 268 J. Biol. Chem. 2458-67; L. Polgar, Mechanisms of Protease Action (1989); Brown, McGuire and Thiele, "Dipeptidyl Peptidase I is Enriched in Granules of In Vitro- and In Vivo-Activated Cytotoxic T Lymphocytes," 150 Immunology 4733-42. The Brown et al. study effectively demonstrated the feasibility of inhibiting cathepsin C (DPP-I) in the presence of other cysteinyl enzymes based on substrate specificity. Unfortunately, the diazoketones used in that study are believed to be mutagenic and not appropriate for in vivo application.

The cytosolic or membrane-bound cysteine proteases called calpains have also been implicated in a number of disease states. For example, calpain inhibitor can be useful for the treatment of muscular disease such as muscular dystrophy, amyotrophy or the like, 25 Taisha (Metabolism) 183 (1988); 10 J. Pharm. Dynamics 678 (1987); for the treatment of ischemic diseases such as cardiac infarction, stroke and the like, 312 New Eng. J. Med. 159 (1985); 43 Salshin Igaku 783 (1988); 36 Arzneimittel Forschung/Drug Research 190, 671 (1986); 526 Brain Research 177 (1990); for improving the consciousness disturbance or motor disturbance caused by brain trauma, 16 Neurochemical Research 483 (1991); 65 J. Neurosurgery 92 (1986); for the treatment of diseases caused by the demyelination of neurocytes such as multiple

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sclerosis, peripheral nervous neuropathy and the like, 47 J. Neurochemistry 1007 (1986); and for the treatment of cataracts, 28 Investigative Ophthalmology & Visual Science 1702 (1987); 34 Experimental Eye Research 413 (1982); 6 Lens and Eye Toxicity Research 725 (1989); 32 Investigative Ophthalmology & Visual Science 533 (1991).

Calpain inhibitors may also be used as therapeutic agents for fulminant hepatitis, as inhibitors against aggregation of platelet caused by thrombin, 57 Thrombosis Research 847 (1990); and as a therapeutic agent for diseases such as breast carcinoma, prostatic carcinoma or prostatomegaly, which are suspected of being caused by an abnormal activation of the sex hormone receptors.

Certain protease inhibitors have also been associated with Alzheimer's disease. See, e.g., 11 Scientific American 40 (1991). Further, thiol protease inhibitors are believed to be useful as anti-inflammatory drugs, 263 J. Biological Chem. 1915 (1988); 98 J. Biochem. 87 (1985); as antiallergic drugs, 42 J. Antibiotics 1362 (1989); and to prevent the metastasis of cancer, 57 Seikagaku 1202 (1985); — Tumor Progression and Markers 47 (1982); and 256 J. Biological Chemistry 8536 (1984).

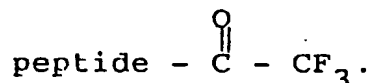
Further, cysteine protease has been shown to process the convertase enzyme of interleukin-1, a cytokine that is implicated in septic shock, wound healing, and the growth of certain leukemias. See, e.g., Thornberry et al., 356 Nature 755; Goll et al., 74 Biochim 225 (1992), and Bioworld Today, Vol. 3, No. 67, p. 1, April 3, 1992.

Although a number of cysteine proteinase inhibitors have been identified, most of these have drawbacks for in vivo use. In particular, drawbacks such as reversibility of inhibition, lack of specificity, and rapid clearance from the body have been associated with prior art inhibitors. The microbial products antipain and leupeptin, for example, are effective but reversible inhibitors of cysteine proteinase

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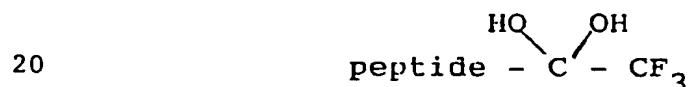
(McConnell et al., 33 J. Med. Chem. 86-93; Sutherland et al. 110 Biochem. Biophys. Res. Commun. 332-38), and also inhibit certain serine proteinases (Umezawa, 45 Meth. Enzymol. 678-95). The compound E64 and its synthetic analogues are  
 5 more selective inhibitors (see, e.g., Barret et al., 201 Biochem. J. 189-98, and Grinde, 701 Biochem. Biophys. Acta. 328-33), but disappear too quickly from the circulation for in vivo use (Hashida et al. 91 J. Biochem. 1373-80).

To date, two classes of peptidyl ketone inhibitors have  
 10 been identified. One class, originally described by Abeles, comprises reversible inhibitors of both serine and cysteine proteases. For example, Abeles et al. describe trifluoromethyl ketones of the form:

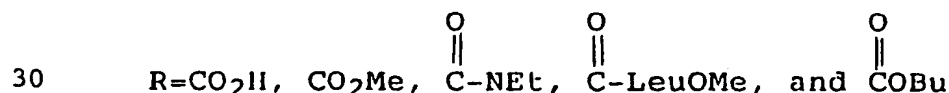
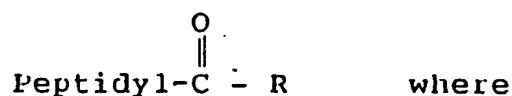


15 Abeles et al., "Fluoroketone Inhibitors of Hydrolytic Enzymes," 24 Biochemistry 1813-17, (1985).

Similarly, reversible inhibitors to serine proteases also exist in their hydrated form:



and therefore become transition state analog inhibitors. Further, Hu et al., "Inhibition of Cathepsin B and Papain by Peptidyl  $\alpha$ -ketoEsters,  $\alpha$ -ketoAmides,  $\alpha$ -Diketones, and  $\alpha$ -ketoAcids", 281 Archives of Biochemistry and Biophysics  
 25 271-274 (1990) describe compounds of similar reactivity of the form:



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Most recently, European Patent publication EP0-525420-A1 disclosed peptidyl ketones that were activated by the permanent (nonfissionable) placement of methylthiomethyl ring or amine, thus making reversible inhibitors of reduced efficacy.

The difference between these noncleavable reversible inhibitors and the irreversible inhibitors of the present invention is that the former inhibitors remain intact in the active site constantly partitioning with the external medium while the latter cleaves and leaves the peptide portion irreversibly bonded to the enzymes active site, thereby permanently disabling the enzyme. This latter mechanism translates into lower required doses of therapeutic agent.

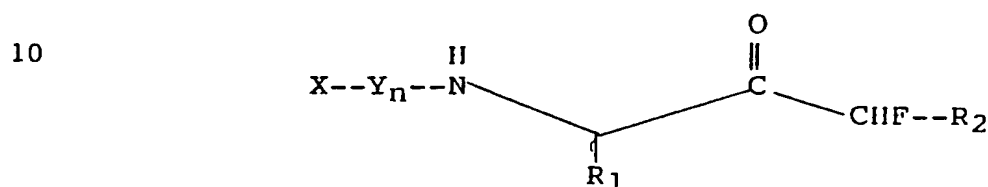
The most promising type of cysteine proteinase inhibitors have an activated carbonyl with a suitable  $\alpha$ -leaving group fused to a programmed peptide sequence that specifically directs the inhibitor to the active site of the targeted enzyme. Once inside the active site, the inhibitor carbonyl is attacked by a cysteine thiolate anion to give the resulting hemiacetal, which collapses via a 1,2-thermal migration of the thiolate and subsequent displacement of the  $\alpha$ -keto-leaving group. The bond between enzyme and inhibitor is then permanent and the enzyme is irreversibly inactivated.

The usefulness of an inhibitor in inactivating a particular enzyme therefore depends not only on the "lock and key" fit of the peptide portion, but also on the reactivity of the bond holding the  $\alpha$ -leaving group to the rest of the inhibitor. It is important that the leaving group be reactive only to the intramolecular displacement via a 1,2-migration of sulfur in the breakdown of the hemithioacetal intermediate.

Groundbreaking work regarding cysteine proteinase inhibitors having an activated carbonyl, a suitable

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$\alpha$ -leaving group and a peptide sequence that effectively and specifically directs the inhibitor to the active site of the targeted enzyme was disclosed in U.S. Patent 4,518,528 to Rasnick, incorporated herein by reference. That patent  
 5 established peptidyl fluoromethyl ketones to be unprecedented inhibitors of cysteine proteinase in selectivity and effectiveness. The fluoromethyl ketones described and synthesized by Rasnick included those of the formula:



wherein  $\text{R}_1$  and  $\text{R}_2$  are independently selected from the group hydrogen, alkyl of 1-6 carbons, substituted alkyl of  
 15 1-6 carbons, aryl, and alkylaryl where the alkyl group is of 1-4 carbons;  $n$  is an integer from 1-4 inclusive;  $\text{X}$  is a peptide end-blocking group; and  $\text{Y}$  is an amino acid or peptide chain of from 1-6 amino acids.

Peptidylketone inhibitors using a phenol leaving group  
 20 are similar to the peptidyl fluoroketones. As is known in the art, oxygen most closely approaches fluorine in size and electronegativity. Further, when oxygen is bonded to an aromatic ring these values of electronegativity become even closer due to the electron withdrawing effect of the  $\text{sp}^2$   
 25 carbons. The inductive effect of an  $\alpha$ -ketophenol versus an  $\alpha$ -ketofluoride when measured by the  $\text{pK}_a$  of the  $\alpha$ -hydrogen, appears comparable within experimental error.

Unfortunately, the leaving groups of prior art cysteine  
 30 protease inhibitors have presented problems of toxicity, solubility, etc. For example, the inhibitors disclosed by Krantz et al. in U.S. Patent No. 5,055,451 have been found to be unacceptably toxic when introduced into animals such as

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rabbits or dogs.

A need therefore exists for cysteine protease inhibitors with improved solubility and toxicity profiles, and which are particularly suitable for in vivo use. The present invention  
5 addresses that need.

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SUMMARY OF THE INVENTION

Briefly describing the present invention, there is provided a class of cysteine protease inhibitors which deactivates the protease by covalently bonding to the  
5 cysteine protease and releasing a heterocyclic leaving group. The cysteine protease inhibitors of the present invention accordingly comprise a first portion which targets a desired cysteine protease and positions the inhibitor near the thiolate anion portion of the active site of the  
10 protease, and a second portion which covalently bonds to the cysteine protease and irreversibly deactivates that protease by providing a carbonyl or carbonyl-equivalent which is attacked by the thiolate anion of the active site of the cysteine protease to sequentially cleave a heterocyclic  
15 leaving group. The heterocyclic leaving group of the protease inhibitor is of the formula: - O - Het, where Het is a heterocycle having 4-7 members in the ring, with at least one of the heterocycle members being N, O or S.

One object of the present invention is to provide  
20 improved cysteine protease inhibitors with improved solubility and toxicity profiles.

A further object of the present invention is to provide a class of cysteine protease inhibitors which are particularly effective for in vivo applications.

25 Further objects and advantages of the present invention will be apparent from the following description.

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DESCRIPTION OF THE PREFERRED EMBODIMENT

For the purposes of promoting an understanding of the principles of the invention, reference will now be made to preferred embodiments and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of the invention is thereby intended, such alterations and further modifications of the invention, and such further applications of the principles of the invention as illustrated herein, being contemplated as would normally occur to one skilled in the art to which the invention relates.

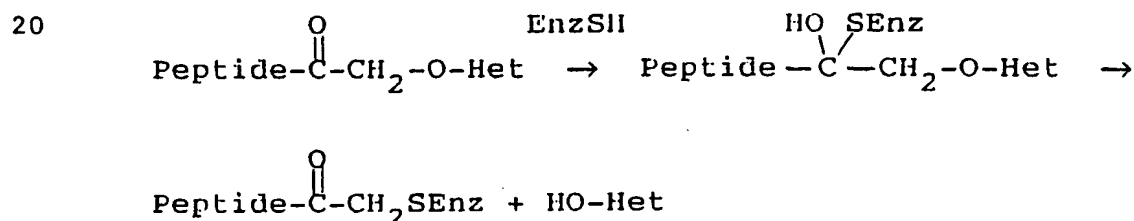
As indicated above, the present invention relates to cysteine protease inhibitors which contain heterocyclic leaving groups. In one aspect of the invention, a group of cysteine protease inhibitors which have been shown to be particularly effective for in vivo applications is disclosed.

The cysteine protease inhibitors described herein function as the sum of two portions. The first portion defines the specificity of a particular inhibitor to an enzyme by the spacial, hydrophobic or hydrophilic and ionic interactions of a particular composition that either imitates or improves upon the nature of the enzyme's natural substrate. The second portion is a trap that covalently binds the enzyme in a two-step mechanism: the first step involves the nucleophilic attack of the enzyme thiolate on the carbonyl of the inhibitor to form a hemithioaketal. It is then energetically favorable for this intermediate to undergo a 1,2 migration of the thiolate in which a heterocyclic leaving group is simultaneously released. The enzyme has now become irreversibly bonded to the inhibitor. With the inhibitors of the present invention the leaving group is a heterocyclic leaving group.

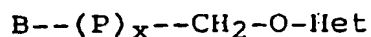
Accordingly, the cysteine proteinase inhibitors of the

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present invention are preferably constructed with an activated carbonyl which bears a suitable  $\alpha$ -leaving group which is fused to a programmed peptide sequence that specifically directs the inhibitor to the active site of the targeted enzyme. (For example, Z-Phe-PheCHN<sub>2</sub> preferentially inhibits cathepsin L over cathepsin B.) Once inside the active site, this inhibitor carbonyl is attacked by a cysteine thiolate anion to give the resulting hemiacetal form. If the  $\alpha$ -leaving group then breaks off, the bond between enzyme and inhibitor becomes permanent and the enzyme is irreversibly inactivated. The selectivity of the inhibitor for a particular enzyme depends not only on the "lock and key" fit of the peptide portion, but also on the reactivity of the bond binding the leaving group to the rest of the inhibitor. It is very important that the leaving group must be reactive only to the intramolecular displacement via a 1,2-migration of sulfur in the breakdown of the hemithioacetal intermediate. The mechanism of protease inhibition is shown below in FIG. 1.



The preferred inhibitors of the present invention can be described generally by the formula:



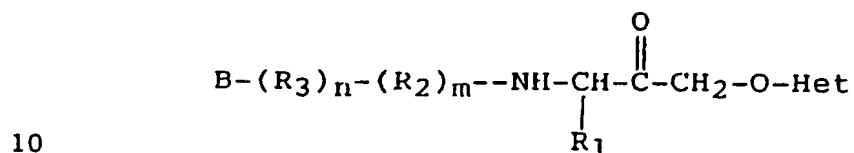
wherein B is an amino acid blocking group for the N-terminal amino acid, each (P)<sub>x</sub> is an optionally protected  $\alpha$ -amino acid residue, x is an integer between zero and 5, inclusive, and Het is the heterocyclic portion of the leaving group.

As is conventional in the art, and as used herein, amino acid residues may be designated as P<sub>1</sub>, P<sub>2</sub>, etc., wherein

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$P_1$  refers to the amino acid residue nearest the leaving group,  $P_2$  refers to the amino acid residue next to  $P_1$  and nearer the blocking group, etc. In dipeptide inhibitors therefore,  $P_2$  is the amino acid residue nearest the  
 5 blocking group.

More specifically, the compounds employed in the present invention are of the formula:



wherein

B is H or an amino acid blocking group for an N-terminal amino acid nitrogen;

$R_1$  is the amino acid side chain of the  $P_1$  amino acid  
 15 residue;

$R_2$  is the amino acid residue of the  $P_2$  amino acid;

$R_3$  is the amino acid residue of the  $P_3$  amino acid;

n is 0 or 1;

m is 0 or 1; and

20 Het is the heterocyclic portion of the leaving group; wherein the heterocyclic leaving group includes a four-, five-, six- or seven-membered ring having at least one C and at least one of N, O or S in the ring.

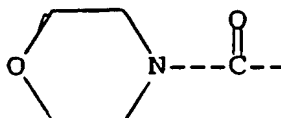
Concerning the amino acid blocking group B for the  
 25 N-terminal amino acid nitrogen, many suitable peptide end-blocking groups are known in the art. For example the end-blocking groups identified in E. Gross and J. Meienhofer (eds.), The Peptides, Vol. 3 are generally suitable for use in the present invention. Preferred blocking groups include  
 30 N-morpholine carbonyl and derivatives of propionic acid derivatives that have intrinsic analgesic or anti-inflammatory action. Examples of blocking groups having intrinsic analgesic or anti-inflammatory action may be found in Gilman, Goodman, Gilman, The Pharmacological Basis of  
 35 Therapeutics, Sixth Ed. MacMillan, Chapter 29. As defined

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herein, the peptide end-blocking group is attached to either an amino acid or a peptide chain.

One particularly effective blocking group is the 4-morpholinylcarbonyl ("Mu") blocking group shown below:

5



4-morpholinylcarbonyl

The peptide portion of the inhibitor may include any peptide appropriate for targetting a desired cysteine protease. Preferably, the side chain on the  $P_1$  amino acid is selected according to the enzyme being targetted. For cathepsin B or L, this might include side chains such that the linked  $P_1$  amino acid is a member of the group consisting of alanyl (Ala), arginyl (Arg), aspartic acid (Asp), glutamic acid (Glu), histidyl (His), homophenylalanyl (HPhe), phenylalanyl (Phe), ornithyl (Orn), seryl (Ser) and threonyl (Thr), and optionally substituted analogues thereof such as thiazoles and amino thiazoles. Preferably the side chain on the  $P_2$  amino acid is selected so that the linked  $P_2$  amino acid is a member of the group consisting of phenylalanyl (Phe), leucyl (Leu), tyrosyl (Tyr) and valyl (Val) amino acid residues and substituted analogues thereof, particularly including Tyr(OMe).

As indicated above, each of the cysteine protease inhibitors of the present invention includes a heterocyclic leaving group. The heterocycle of the leaving group is a ring of variable size (4-7 members) containing one or more heteroatoms and  $n$  double bonds where  $n = 0-3$ . This ring is bonded through an oxygen atom which becomes part of the leaving group during inhibition of the targeted enzyme. In the absence of the targeted active site, the inhibitor construction is an ether that is very stable in the physiological environments of the stomach, lysosome, or

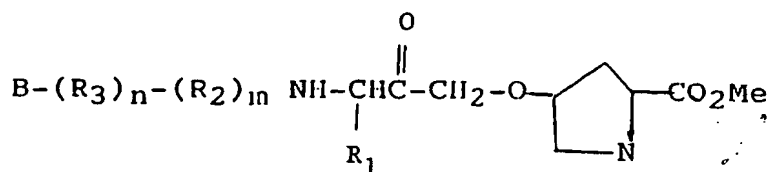
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disease state arthritic joint.

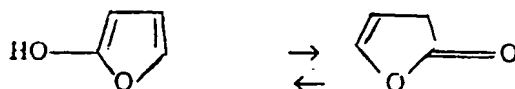
The heterocycles of the present invention may be derived from the group that includes pyridine n-oxides, uracils, cytosines, pyrones, pyridones, pyrimidines, pyrazines, furans, thiophenes, pyrroles, oxazoles, thiazoles, pyrazoles, imidazoles, triazoles, tetrazoles and their optionally-substituted derivatives such as benzo-fused derivatives. For oral bioavailability, the most preferred leaving groups have at least one heteroatom for each four carbon atoms in the leaving group.

The ability of the leaving group to protonate at physiological pH is directly related to its inability to cross cell membranes which can be advantageous in certain applications. In the same context, the ability of a heterocycle to chelate with a metal ion may (1) impede crossing of membranes and/or (2) target metalloproteases such as calpains. In the environment of the active site protonation and/or hydrogen bonding of ring heteroatoms will accentuate its role as a leaving group.

It is to be appreciated that the heterocycle may be chosen specifically in accord with the function of the enzyme to be inhibited. For example, the inhibitor would use a derivative of hydroxyproline (a major constituent of collagen) to target a collagenase:



or the natural substrate (hydroxyproline) may be improved upon by a suitable replacement such as a hydroxyfuran which upon displacement from the inhibitor can and does tautomerize to a more stable keto form increasing its efficiency as a leaving group.



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It is also to be appreciated that during the course of the reaction of enzyme with inhibitor, the carbonyl of the inhibitor rehybridizes to  $sp^3$  and forms a ketal intermediate with the thiol function of the enzyme. Under the acid  
5 conditions of this reaction, other ketals can exchange with this intermediate either by going through the ketone or by ketal-ketal exchange. Accordingly, ketals can be substituted for carbonyls in the peptidyl inhibitors of the present invention. Similarly, other compounds such as hydrazones,  
10 hemiketals, oximes, imines, cyanohydrins, enolethers, enamines, hemithioketals, and the like are to be considered carbonyl equivalents and may be substituted for a carbonyl or to give a carbonyl under the acidic conditions of these inhibition reactions. Utilization of such derivatives can  
15 also be vehicles to either improve the bioavailability of the inhibitor drug or keep it from crossing a cellular membrane depending upon the hydrophobic nature of the masking function.

Finally, it is to be appreciated that the development and synthesis of compounds having isosteric replacements of amide  
20 bonds is now a standard practice in the development of biologically active peptides once the optimum peptide sequence has been identified. Accordingly, the present invention includes compounds having one or more modified amide bonds in the peptide sequence so long as conformation  
25 and binding are maintained while secondary enzymatic hydrolysis is prevented. For a list of such modifications see Kaltenbronn, 33, J. Med. Chem., 838. In addition, inhibitors having a hydrazine replacement for the  $P_1$  nitrogen as reported by Giordano for other halogen methyl  
30 ketones are also intended to be claimed.

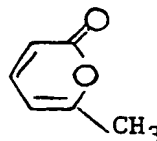
Reference will now be made to specific examples using the processes described above. It is to be understood that the examples are provided to more completely describe preferred

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embodiments, and that no limitation to the scope of the invention is intended thereby.

## EXAMPLE 1

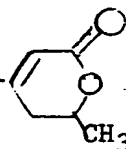
5

Synthesis of Mu-Phe-HPhe-CH<sub>2</sub>-O-

In a 100 ml round bottom flask equipped with an argon line was placed Mu-Phe-HPhe-Br (1.0 g, 1.94 mmol), potassium fluoride (449.5 mg, 7.75 mmol), potassium carbonate (534.9 mg, 3.87 mmol)-K<sub>2</sub>CO<sub>3</sub> was added to control the acidic environment, and 4-hydroxy-6-methyl-2-pyrone (488.0 mg, 3.87 mmol). About 5 ml of DMF was added to dissolve the solid mixture. The reaction flask was immersed in a 50°C oil bath. The reaction was allowed to run for 40 minutes in order to ensure the completion of the reaction. The reaction mixture was diluted with ethyl acetate, and potassium fluoride was removed by a small silican gel column. The solvent ethyl acetate was stripped by a water vacuum pump, and DMF was removed by an oil vacuum pump. The next day the light yellow product was recrystallized in hexane:diethyl ether (50%:50%). The white precipitate was filtered and a NMR spectrum was obtained. m.p. 94-98°C.

## EXAMPLE 2

25

Synthesis of Mu-Phe-HPhe-CH<sub>2</sub>-O-

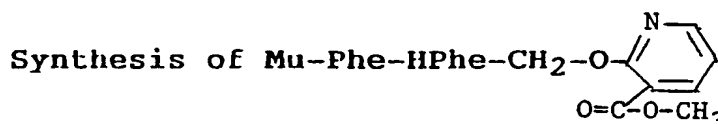
In a 100 ml round bottom flask equipped with an argon line was placed Mu-Phe-HPhe-Br (300 mg, 0.581 mmol), potassium fluoride (134.8 mg, 2.33 mmol), potassium carbonate (321.4 mg, 2.33 mmol)-K<sub>2</sub>CO<sub>3</sub> was added to control the acidic environment, and 5,6-dihydro-4-hydroxy-6-methyl-2H-pyran-2-one (298.0 mg, 2.33 mmol). About 5 ml of DMF was added to dissolve the solid mixture. The reaction flask was immersed in a 50°C oil

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bath. The reaction was allowed to run for 40 minutes in order to ensure the completion of the reaction. The reaction mixture was diluted with ethyl acetate, and potassium fluoride was removed by a small silica gel column. The solvent ethyl acetate was removed by a water vacuum pump, and DMF was removed by an oil vacuum pump. The next day the light yellow product was recrystallized in 50:50 hexane:diethyl ether. The white precipitate was filtered and a NMR spectrum was obtained. m.p. 74-78°C.

10

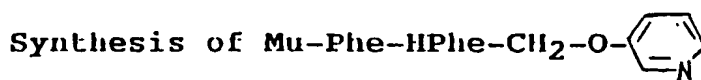
## EXAMPLE 3



In a 100 ml round bottom flask equipped with an argon line was placed Mu-Phe-HPhe-Br (1 g, 1.94 mmol), potassium fluoride (0.45 g, 7.75 mmol), potassium carbonate (1.07 g, 7.75 mmol)-K<sub>2</sub>CO<sub>3</sub> was added to control the acidic environment, and 2-hydroxy-3-methylcarboxypyridine (0.59 g, 3.87 mmol). About 5 ml of DMF was added to dissolve the solid mixture. The reaction flask was immersed in a 50°C oil bath. The reaction was allowed to run for 40 minutes in order to ensure the completion of the reaction. The reaction mixture was diluted with ethyl acetate, and potassium fluoride was removed by a small silica gel column. The solvent ethyl acetate was removed by a water vacuum pump, and DMF was removed by an oil vacuum pump. The next day the light yellow product was recrystallized in 50:50 hexane:diethyl ether. The white precipitate was filtered and a NMR spectrum was obtained. m.p. 100-102°C.

30

## EXAMPLE 4



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In a 100 ml round bottom flask equipped with an argon line was placed Mu-Phe-HPhe-Br (500 mg, 0.969 mmol), potassium fluoride (224.8 mg, 3.876 mmol), potassium carbonate (267.8 mg, 1.938 mmol)-K<sub>2</sub>CO<sub>3</sub> was added to  
5 control the acidic environment, and 3-hydroxypyridine (184.3 mg, 1.938 mmol). About 5 ml of DMF was added to dissolve the solid mixture. The reaction flask was immersed in a 50°C oil bath. The reaction was allowed to run for 40 minutes in order to ensure the completion of the reaction. The reaction  
10 mixture was diluted with ethyl acetate, and potassium fluoride was removed by a small silica gel column. The solvent ethyl acetate was removed by a water vacuum pump, and DMF was removed by an oil vacuum pump. The next day the light yellow product was recrystallized in 50:50  
15 hexane:diethyl ether. The white precipitate was filtered and a NMR spectrum was obtained. m.p. 112-115°C.

## EXAMPLE 5

Synthesis of Mu-Phe-HPhe-CH<sub>2</sub>-O-

In a 100 ml round bottom flask equipped with an argon  
20 line was placed Mu-Phe-HPhe-Br (300 mg, 0.581 mmol), potassium fluoride (135.1 mg, 2.33 mmol), potassium carbonate (322.0 mg, 2.33 mmol)-K<sub>2</sub>CO<sub>3</sub> was added to control the acidic environment, and 2-hydroxypyrimidine (111.7 mg, 1.938 mmol). About 5 ml of DMF was added to dissolve the solid  
25 mixture. The reaction flask was immersed in a 50°C oil bath. The reaction was allowed to run for 40 minutes in order to ensure the completion of the reaction. The reaction mixture was diluted with ethyl acetate, and potassium fluoride was removed by a small silica gel column. The  
30 solvent ethyl acetate was removed by a water vacuum pump, and DMF was removed by an oil vacuum pump. The next day the light yellow product was recrystallized in 50:50

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hexane:diethyl ether. The white precipitate was filtered and a NMR spectrum was obtained. m.p. 95-100°C.

## EXAMPLE 6

Synthesis of Mu-Phe-HPhe-CH<sub>2</sub>-O-

5 In a 100 ml round bottom flask equipped with an argon line was placed Mu-Phe-HPhe-CH<sub>2</sub>-Br (500 mg, 0.969 mmol), potassium fluoride (224.8 mg, 3.876 mmol), potassium carbonate (535.7 mg, 3.876 mmol)-K<sub>2</sub>CO<sub>3</sub> was added to control the acidic environment, and 2-furanone (275  $\mu$ l, 10 3.876 mmol). About 5 ml of DMF was added to dissolve the solid mixture. The reaction flask was immersed in a 50°C oil bath. The reaction was allowed to run for 40 minutes in order to ensure the completion of the reaction. The reaction mixture was diluted with ethyl acetate, and potassium 15 fluoride was removed by a small silica gel column. The solvent ethyl acetate was removed by a water vacuum pump, and DMF was removed by an oil vacuum pump. The next day the light yellow product was recrystallized in 50:50 hexane:diethyl ether. The white precipitate was filtered and 20 a NMR spectrum was obtained. m.p. 90-93°C.

## EXAMPLE 7

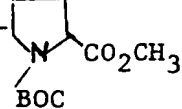
Synthesis of Mu-Phe-HPhe-CH<sub>2</sub>-O-

In a 100 ml round bottom flask equipped with an argon line was placed Mu-Phe-HPhe-Br (200 mg, 0.388 mmol), 25 potassium fluoride (89.9 mg, 1.55 mmol), potassium carbonate (214.3 mg, 1.55 mmol)-K<sub>2</sub>CO<sub>3</sub> was added to control the acidic environment, and 2-hydroxypyrimidine (205.5 mg, 1.55 mmol). About 4 ml of DMF was added to dissolve the solid mixture. The reaction flask was immersed in a 50°C oil 30 bath. The reaction was allowed to run for 40 minutes in

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order to ensure the completion of the reaction. The reaction mixture was diluted with ethyl acetate, and potassium fluoride was removed by a small silica gel column. The solvent ethyl acetate was removed by a water vacuum pump, and  
5 DMF was removed by an oil vacuum pump. The next day the light yellow product was recrystallized in 50:50 hexane:diethyl ether. The white precipitate was filtered and a NMR spectrum was obtained. m.p. 82-85°C.

## EXAMPLES 8-9

- 10 Synthesis of Mu-Phe-HPhe-CH<sub>2</sub>-O-  
(MuPheHPhe-O-(proline methyl ester)).

In a 100 ml round bottom flask equipped with an argon  
15 line was placed Mu-Phe-HPhe-Br (500 mg, 0.969 mmol), potassium fluoride (224.8 mg, 3.876 mmol), potassium carbonate (535.7 mg, 3.876 mmol) was added to control the acidic environment, and Boc-L-hydroxyproline-methylester (475.2 mg, 1.938 mmol). About 6 ml of DMF was added to  
20 dissolve the solid mixture. The reaction was left at the room temperature and periodical TLC was checked to monitor the progress of the reaction. According to TLC, the reaction was completed after about one hour. The reaction mixture was diluted with ethyl acetate, and potassium fluoride was  
25 removed by a short silica gel column. The solvent ethyl acetate was stripped by a water vacuum pump, and DMF was removed by an oil vacuum pump. The product was dried under the vacuum pump. The next day the light yellow oily product was recrystallized in hexane. The precipitate was filtered  
30 and a NMR spectrum was obtained.

De-Boc: About 5 ml of methylene chloride was used to dissolve 100 mg of the Boc product. Then about 5 ml of HCl. Dioxane was put into the 50 ml flask, and the reaction was

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allowed to proceed for about 45 minutes under argon. Carbon dioxide was released from the flask; bubbles came up to the surface of the reaction mixture. After the completion of the reaction, the mixture was slowly dropped into six test tubes  
5 filled with diethyl ether. The precipitation came out and was allowed to continue for about half hour. The product was quickly removed through suction filtration and it was put in a dry box overnight.

## EXAMPLE 10

10 Synthesis of Mu-Phe-HPhe-CH<sub>2</sub>-O-CH<sub>2</sub>CH<sub>3</sub>.

In a 100 ml round bottom flask equipped with an argon line was placed Mu-Phe-HPhe-CH<sub>2</sub>-Br (300 mg, 0.582 mmol), potassium fluoride (150 mg, 2.4 mmol), and potassium carbonate (150 mg, 1.2 mmol), and 8 ml of 200 proof ethanol.  
15 The reaction was stirred 3 hours, filtered and the solvents were removed under vacuum and the residue chromatographed on a short silica gel column (CHCl<sub>3</sub>:MeOH 95:5) and then recrystallized from ether.

## EXAMPLE 11

20 General Procedure for the Preparation of HCl-HPhe-CH<sub>2</sub>-O-Substituted Heterocyclic Inhibitors(a) BOC-HPhe diazoketone

To BOC homophenylalanine (18 mmol) in 150 ml distilled THF at -20°C was added one equivalent of N-methylmorpholine  
25 followed by one equivalent of isobutylchloroformate. After 10 minutes, the mixture was poured through filter paper into 200 ml ethereal solution of diazomethane which was made according to the suppliers directions from 8.6 g of Diazald (Aldrich). The reaction was allowed to stir overnight, and  
30 then poured into 100 ml water. The organic portion was

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washed with  $\text{NaHCO}_3(\text{aq})$  (2X50 ml), brine (2X50 ml); dried over  $\text{MgSO}_4$ ; concentrated to give the diazoketone 5.38 g (99%) of a yellow oil that crystalizes on standing.

(b) BOC-HPhe-CH<sub>2</sub>Br

5 To the above diazoketone (5.38 g, 0.018 mol) in 20 ml of methylene chloride at  $-5^\circ\text{C}$  is added dropwise 30% HBr in acetic acid which is diluted three-fold with methylene chloride. The addition is made at a rate that allows the monitoring of the evolved nitrogen and until all yellow color  
10 of the starting material has disappeared. The reaction is poured into 50 ml water and diluted with another 100 ml of methylene chloride. The organic layer is separated, washed with  $\text{NaHCO}_3$  (aq) (50 ml), brine (50 ml), dried over  $\text{MgSO}_4$  and concentrated to give a white solid which is characterized  
15 by its NMR.

(c) BOC-HPhe-CH<sub>2</sub>-O-Substituted Heterocycle

In a 100 ml round bottom flask equipped with an argon line was placed BOC-HPhe-CH<sub>2</sub>Br (1.4 mmol), potassium fluoride (5.6 mmol) and the substituted phenol (5.6 mmol).  
20 About 1 ml of DMF is added and the mixture is stirred at  $50^\circ\text{C}$  for 40 min. The reaction is then diluted with ethyl acetate and run through a silica gel plug to remove the potassium salts. The solvents are removed (low then high) vacuum to give a solid product. When purification is needed,  
25 a silica gel column is used. In this way the following compounds were prepared:

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Important NMR Signals (100 MHz, CDCl<sub>3</sub>)

<u>Compound</u>	BOC	O	
		C-CH <sub>2</sub> -O	O-Het-R
BOC-HPhe-O-Methyl nicotinate	1.44	4.95	3.875
5 BOC-HPhe-O-furan	1.44	4.8	
BOC-HPhe-O-methyl pyrone	1.44	4.8	2.6

(d) Prep HCl - HPheCH<sub>2</sub>-O-Substituted heterocycle

To the BOC-PHhe CH<sub>2</sub>-O-Substituted Aromatic (2.6mmol) in 3ml of methylene chloride was placed 8ml of 2N HCl -  
 10 dioxane. The reaction was stirred about 40 min and the resulting mixture was added dropwise to about 600ml of ether and then filtered to give the peptide inhibitor.

## EXAMPLES 12-13

15 Evaluation of heterocyclic-methyl, peptidyl ethers by the in vitro inhibition of purified enzymes Cathespin B and H

## Example 12: FLUOROKETONE - CATHESPIN B KINETICS

Enzyme: Cathespin B, purified from human liver, is from Enzyme Systems Products (Dublin, CA). The activity is 50 mU per ml at 30°C, in 52 mM sodium phosphate, pH 6.2, 31 mM DTT,  
 20 2.1 mM EDTA, with 0.2 mM Z-Arg-Arg-7-amino-4-trifluoromethyl-coumarin as a substrate. Specific activity is 8330 mU per mg protein. (1 mU = 1 nmol per min.)

Substrate: Boc-Leu-Arg-Arg-7-amino-4-trifluoromethyl-coumarin-2HBr is from Enzyme Systems Products. A 20 mM  
 25 solution is made in DMF and stored at -20° C.

Inhibitors: Candidate inhibitors are synthesized by

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Prototek, Inc., Dublin, California. 20 mM stock solutions are made in DMF and stored at  $-20^{\circ}\text{C}$ . Dilutions are made in assay buffer.

Method: The percent inhibition and the inhibitor concentration at which the enzyme is 50% inhibited ( $\text{IC}_{50}$ ) are determined as follows:

Five  $\mu\text{l}$  of enzyme are activated by pre-incubation in three 480  $\mu\text{l}$  aliquots and one 485  $\mu\text{l}$  aliquot of assay buffer (50 mM potassium phosphate pH 6.2, 2 mM EDTA, 5 mM DTT) on ice for 30 min. The inhibition is initiated by the addition of 5  $\mu\text{l}$  of 200  $\mu\text{M}$ , 20  $\mu\text{M}$ , and 2  $\mu\text{M}$  inhibitor each to the 480  $\mu\text{l}$  aliquots. The 485  $\mu\text{l}$  aliquot with enzyme is used as a control and thus receives no inhibitor. The enzyme/inhibitor mixtures are incubated 10 min. on ice and assayed for cathepsin B activity as follows.

Cathepsin B assay: To 490  $\mu\text{l}$  of pre-incubated inhibitor/enzyme mixtures in assay buffer in 0.5 ml cuvette at  $37^{\circ}\text{C}$  is added 10  $\mu\text{l}$  of the substrate. Final inhibitor concentrations become 2000 nM, 200 nM, and 20 nM for the 200  $\mu\text{M}$ , 20  $\mu\text{M}$  and 2  $\mu\text{M}$  stock concentrations, respectively. Activity is followed by release of free AFC over 5 min. (where (fluorescence units at  $t=6$ ) - (fluorescence units at  $t=1$ )) with a Perkin-Elmer LS-5B spectrofluorometer (ex = 400 nm, em = 505 nm).

The percent inhibition is determined by comparing the change in fluorescence units of the three sample concentrations of inhibited enzyme to the change in fluorescence units of the control enzyme.  $100 - (\text{fl. units of sample} / \text{fl. units of control} \times 100)$  gives percent inhibition.

The  $\text{IC}_{50}$  is ascertained by plotting percent inhibition vs. inhibitor concentration on the log scale. The  $\text{IC}_{50}$  is the concentration of inhibitor (nM) at which the enzyme is inhibited by 50%.

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ACTIVITY AGAINST CATHESPIN B (in vitro)

Inhibitor (Example number)	rel. (in vitro)
MuPhe HPhe CH <sub>2</sub> -O-(methyl)pyrone (1)	112.2 nM
MuPhe HPhe CH <sub>2</sub> -O-(methyl)dihydro pyrone (2)	567.5 nM
5 MuPhe HPhe CH <sub>2</sub> -O-(methyl)nicotinate (3)	25,100.0 nM
MuPhe HPhe CH <sub>2</sub> -O-(3)-pyridine (4)	66.8 nM
MuPhe HPhe CH <sub>2</sub> -O-(4)-pyrimidine (5)	7,100.0 nM
MuPhe HPhe CH <sub>2</sub> -O-(2)-furan (6)	23.7 nM
MuPhe HPhe CH <sub>2</sub> -O-(2)-pyrimidine (7)	631.0 nM
10 MuPhe HPhe CH <sub>2</sub> -O-proline methyl ester	
N-Boc (8)	42.2 nM
N-HCl (9)	223.9 nM

## Example 13

## FLUOROKETONE - CATHESPIN H KINETICS

- 15      The materials and methods were as disclosed above for Example 12 - cathepsin B kinetics - except as follows:

Enzyme: Cathepsin H

Substrate: HBr-Arg-7-amino-4-trifluoromethyl-coumarin

Methods: assay buffer is pH 6.8

20                      Activity Against Cathepsin H

HCl · HPhe-O-(methyl)nicotinate	10.0 nM
HCl · HPhe-O-(methyl)pyrone	150.0 nM

## EXAMPLES 14-15

Testing Heterocycle and Phenolic Leaving Groups In Vivo

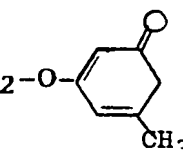
- 25      Water solutions of inhibitor using either ethanol or DMSO

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as a cosolvent were made at 8000 nM, 800 nM and 160 nM concentrations with a minimum of solvent. Each rat was given a dose at time 0 and then individuals were sacrificed at 6 hrs, 12 hrs and 24 hrs. Doses were given either by injection or stomach tube.

The livers and kidneys were harvested and analyzed for Cathespin B activity. In some individuals the urine was collected at 6, 12, 24 hrs and analyzed for the presence of inhibitor. The results for Examples 14 and 15 are shown in 10 Tables 14 and 15 below.

TABLE 14


Inhibition of Cathespin B (in vivo) of MuPheHPhCH<sub>2</sub>-O-

	<u>Animal</u>	<u>Hrs</u>	<u>Dose</u>	<u>Route</u>	<u>%Inh.</u>	<u>% Inhb Paired Animal</u>	<u>Mean %Inhb</u>
15	1	24	contr.	inject.			
	2	24	contr.	st			
	3	6	8000	inject.	99.6		99.6
20	4	6	800	inject.	59.0	43.60	51.3
	5	6	160	inject.	10.3	31.80	21.0
	6	6	8000	st.	89.2		90.4
	7	6	800	st.	25.2		25.7
	8	6	160	st.	23.9	94.50	59.2
25	9	12	8000	inject.	89.3		90.5
	10	12	800	inject.	43.5	54.80	49.1
	11	12	160	inject.	16.4	39.10	27.8
	12	12	8000	st.	46.5	57.70	52.1
	13	12	800	st.	9.6		11.6
30	14	12	160	st.	15.9		14.8
	15	24	8000	inject.	94.9	64.70	79.8
	16	24	800	inject.	40.9	57.40	49.2
	17	24	160	inject.	35.9	23.30	29.6
	18	24	8000	st.	66.8		64.0
35	19	24	800	st.	22.8	97.50	60.2
	20	24	160	st.	99.6		99.2

The results shown in Table 14 demonstrate effective inhibition and long duration of action.

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TABLE 15

Inhibition of Cathepsin B In Vivo using  $\text{MuPheHPheCH}_2\text{O}-$ 

	<u>Time (hrs)</u>	<u>Dose</u>	<u>Route</u>	<u>%Inhibition</u>
5	6	8,000	injection	63.9
	6	800	injection	46.9
	6	160	injection	35.6
10	12	8,000	injection	46.2
	12	800	injection	32.7
	12	160	injection	10.1
	24	8,000	injection	62.7
	24	800	injection	53.5
	24	160	injection	42.6
15	6	8,000	st.	79.9
	6	800	st.	54.1
	6	160	st.	61.2
	12	8,000	st.	83.7
	12	800	st.	65.7
	12	160	st.	61.6
20	24	8,000	st.	49.3
	24	800	st.	49.2
	24	160	st.	40.7

The results shown in Table 15 demonstrate effective inhibition with long duration of action.

## EXAMPLE 16

### 25 Treatment of Rheumatoid Arthritis with Mu-Phe-HPhe-O-proline methyl ester

DBA/Lac mice were injected with 200  $\mu\text{g}$  of type II chick collagen emulsified in Freund's complete adjuvant on day 0 and on day 21. Mu-Phe-HPhe-O-proline methyl ester was  
 30 suspended in an ethanol solution which was then diluted to 10% (aq) and administered by gavage at a dose of 10 mg/kg/day from day 21 until sacrifice at day 49. The severity of joint

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inflammation was evaluated grossly at seven day intervals beginning on day 21.

- The effect of oral administration of Mu-Phe-HPhe-O-proline methyl ester on the severity of bone lesions in adjuvant-induced arthritis was determined by evaluating osseous mineralization, periostat proliferation, bone erosion, joint space narrowing and osseous fragmentation. All lesions were scored on a scale of 0 (normal architecture) to 3 (severe or marked changes).
- Values calculated were mean values  $\pm$  std. error of the mean. The "bone lesion severity" values of Mu-Phe-HPhe-O-proline methyl ester treated animals were significantly ( $p < 0.05$ ) lower than the values of control animals for each parameter tested.
- The effect of oral administration of Mu-Phe-HPhe-O-proline methyl ester on the histological aspects of adjuvant-induced arthritis was determined by evaluating inflammation, focal ulcers, tibiotarsal joint cartilage destruction, bone destruction and periosteal proliferation. Inflammation was scored on a scale of 0 (no inflammation) to 3 (severe) based on the extent of edema and cell infiltration. Focal ulcer cartilage destruction was measured as the percent of articular cartilage surfaces with focal destruction which exposed underlying subchondral bone.
- Tibiotarsal joint cartilage destruction was measured as the percent of articulating surfaces with destruction of the subchondral bone. Values calculated were mean values  $\pm$  1 std. error of the mean. The "histological aspects" values of Mu-Phe-HPhe-O-proline methyl ester treated animals were significantly ( $p < 0.05$ ) lower than the values of control animals for each parameter tested.

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## EXAMPLE 17

**Treatment of Rheumatoid Arthritis with Mu-Leu-HPhe-O-proline methyl ester**

DBA/Lac mice were injected with 200 µg of type II chick  
5 collagen emulsified in Complete Freund's Adjuvant on day 0  
and on day 21. Mu-Phe-HPhe-O-proline methyl ester was  
suspended in phosphate buffered saline and administered by  
gavage from day 21 until sacrifice at day 35. Doses of  
between 3 mg/kg/day and 25 mg/kg/day were used, with the  
10 daily dosage being held constant over time for each test.  
The severity of joint inflammation was evaluated grossly as  
seven day intervals beginning on day 21.

The effect of oral administration of  
Mu-Phe-HPhe-O-proline methyl ester on the severity of bone  
15 lesions in adjuvant-induced arthritis was determined by  
evaluating osseous mineralization, periostat proliferation,  
bone erosion, joint space narrowing and osseous  
fragmentation. All lesions were scored on a scale of 0  
(normal architecture) to 3 (severe or marked changes). Values  
20 calculated were mean values  $\pm$  1 std. error of the mean. The  
"bone lesion severity" values of Mu-Phe-HPhe-O-proline methyl  
ester treated animals were significantly ( $p < 0.05$ ) lower  
than the values of control animals for each parameter tested.

## EXAMPLE 18

**25 Treatment of Rheumatoid Arthritis with Mu-Tyr(OMe)-HPhe-O-proline methyl ester**

Rates were injected with adjuvant on day 0 and were  
treated with Mu-Tyr-(OMe)-HPhe-O-proline methyl ester in  
ground diet from the time of adjuvant injection until  
30 sacrifice at day 32. Over the course of the disease, animals

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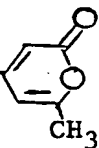

were evaluated in terms of a mean clinical score, a derived series of clinically observed parameters normalized to facilitate graphical presentation. Mean paw volumes were also measured using normalized units throughout the course of the disease. At the end of the study, the animal groups were sacrificed and evaluated by X-ray analysis.

The mean clinical scores of Mu-Tyr-(OMe)-HPhe-O-proline methyl ester treated rats were significantly lower than the scores for untreated animals. Similarly, mean paw volumes were also significantly reduced. The Example demonstrates the beneficial effect of Mu-Tyr-(OMe)-HPhe-O-proline methyl ester on adjuvant-induced arthritis.

## EXAMPLE 19

### Effectiveness of Heterocyclic Methyl Ketones on Human and Murine Malaria Paracytes

In Vitro Effectiveness of Heterocyclic Methyl Ketones  
(All assays with Z-Phe-Arg-AMC)

		IC50 VALUES	
		P. falciparum (Human malaria Paracyte)	P. vinckei (Mouse malaria Paracyte)
20	MuPheHPheCH <sub>2</sub> -O- 	15 nM	40 nM
	MuPheHPheCH <sub>2</sub> -O- 	50 nM	500 nM
25	MuPheHPheCH <sub>2</sub> -O- 	2 μM	10 μM

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## EXAMPLE 20

## Treatment of Gingivitis with MU-Phe-HPhe-O-furan

The effect of the inhibitor on gingival inflammation was studied by investigating its ability to prevent the development of experimental gingivitis. Twenty human subjects with healthy gingiva and no periodontal disease were recruited. The subjects received oral hygiene instruction and scaling prior to baseline. The test sites were the mesibuccal crevices on the upper first and second molars and premolars (bicuspid). Acrylic shields were made to cover the gingival margins of these teeth and were worn during oral hygiene to prevent brushing of the test and control sites. During the test period of three weeks the subjects were told to brush only the lower teeth and upper anteriors and to wear the shields during brushing. The left side was used as the test side and the right was the control side in 10 subjects. The sides were reversed in the other 10 subjects so the subjects could act as their own controls. Thirty second GCF samples were taken prior to clinical measurements at baseline and at 1, 2, 3 and 4 weeks. These were assayed for cathespins B and L-like activities. Clinical measurements of gingival index (GI), gingival bleeding index (GBI) and plaque index (PLI) were taken at test and control sites at zero, one, two, three and four weeks. Following the baseline measurements the inhibitor and placebo were placed at test and control sites and sealed in with Coe-pak for a week. The inhibitor and placebo were coded so that the study was blind.

The GI, GBI and PLI and cysteine proteinase levels were compared at test and control sites. It was observed that the use of Mu-Phe-HPhe-O-furan reduced all parameters tested at the test sites. Mu-Phe-HPhe-O-furan can therefore be seen to be effective in treating periodontal diseases such as

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gingivitis.

In the following Examples 21-27, the active ingredient is the compound morpholine carbonyl-L-phenylalanyl-L-homophenyl alanyl methyl furanyl ether (see Example 6). However, other compounds of the present invention can be substituted thereof.

#### EXAMPLE 21

An injectable preparation buffered to a pH of 7 is prepared having the following composition

10	<u>Ingredients:</u>	Active ingredient	02 g
		KH <sub>2</sub> PO <sub>4</sub> buffer	2 ml
		(0.4 M solution)	
		KOH (1N)	q.s. to pH 7
		Water (distilled, sterile)	q.s. to 20 ml

#### EXAMPLE 22

15 An oral suspension is prepared having the following composition

20	<u>Ingredients</u>	Active ingredient	0.1 g
		fumaric acid	0.5 g
		sodium chloride	2.0 g
		methyl paraben	0.1 g
		granulated sugar	25.5 g
		sorbitol (70% solution)	12.85 g
		Veegum K (Vanderbilt Co.)	1.0 g
		flavoring	0.035 ml
		colorings	0.5 mg
		distilled water	q.s. to 100 ml

#### EXAMPLE 23

	<u>Ingredients</u>	<u>Quantity per tablet, mgs.</u>
30	Active ingredient	25
	cornstarch	20
	lactose, spray-dried	153
	magnesium stearate	2

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## EXAMPLE 24

	<u>Ingredients</u>	<u>Quantity per tablet, mgs.</u>
5	Active ingredient	100
	lactose, spray-dried	148
	magnesium stearate	2

## EXAMPLE 25

	<u>Ingredients</u>	<u>Quantity per tablet, mgs.</u>
10	Active ingredient	200
	cornstarch	50
	lactose	145
	magnesium stearate	5

The above ingredients are mixed intimately and pressed into single scored tablets.

## EXAMPLE 26

	<u>Ingredients</u>	<u>Quantity per tablet, mgs.</u>
15	Active ingredient	108
	lactose	15
	cornstarch	25
	magnesium stearate	2

20 The above ingredients are mixed and introduced into a hard-shell gelatine capsule.

## EXAMPLE 27

	<u>Ingredients</u>	<u>Quantity per tablet, mgs.</u>
25	Active ingredient	150
	lactose	90

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The above ingredients are mixed and introduced into a hard-shell gelatine capsule.

## TOPICAL FORMULATION

	<u>Ingredients</u>	<u>Grams</u>
5	Active compound	0.2-2
	Span 60	2
	Tween 60	2
	Mineral oil	5
	Petrolatum	10
10	Methyl paraben	0.15
	Propyl paraben	0.05
	BHA (butylated hydroxy anisole	0.01
	Water	q.s. 100

All of the above ingredients, except water, are combined and heated to 60°C with stirring. A sufficient quantity of water at 60°C is then added with vigorous stirring to emulsify the ingredients, and water then added q.s. 100 g.

As previously indicated, the compositions of the present invention may be useful in treating disease states which are associated with cathepsins B, L, H or C. For example, the cathepsin C inhibitors of the present invention may be used to inhibit dipeptidyl peptidase I ("DPP-I") found in cytotoxic T lymphocytes, or to inhibit the processing enzyme of bone marrow serine proteases like elastase and granzyme A.

It is to be appreciated that vinylogous homologs of heterocycles (benzo-derivatives) would also be considered by those skilled in the art to be reactive in this context. For example, because a hydroxy pyridine derivative works as a leaving group, a hydroxyquinoline (5, 6, 7 or 8-hydroxy) would also work. Other examples of this principle of vinylogy in synthetic organic chemistry include:

- (1) A Michael reaction is a vinylogous analog of an aldol condensation; and
- (2) An  $SN_2$  reaction is a vinylogous analog to an  $SN_2'$  reaction.

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It is also to be appreciated that because sulfur falls below oxygen in the periodic table that S may replace O in the compositions and methods of the present invention and an effective cysteine protease inhibitor would result.

5        Finally, it is to be appreciated that the processing of viral proteins by virally encoded proteases plays a central role in the maturation of many viruses. Examples of viruses which have been associated with cysteine proteases are polio virus (3C proteases) encephalomyocarditis virus, rhinovirus  
10 and foot-and-mouth virus. Accordingly, the compositions of the present invention are believed to be useful therapeutic agents for the treatment of viral diseases.

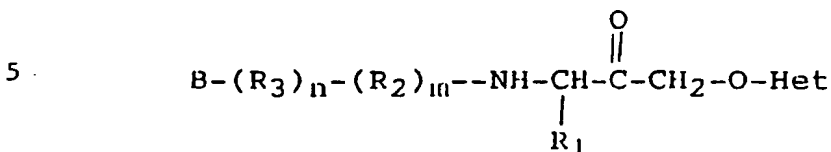
While the invention has been illustrated and described in detail in the drawings and foregoing description, the same is  
15 to be considered as illustrative and not restrictive in character, it being understood that only the preferred embodiment has been shown and described and that all changes and modifications that come within the spirit of the invention are desired to be protected.

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## CLAIMS

What is claimed is:

1. Cathepsin inhibitors of the formula:



wherein

B is H or an amino acid blocking group for an N-terminal amino acid nitrogen;

- 10  $\text{R}_1$  is the amino acid side chain of the  $\text{P}_1$  amino acid residue;

$\text{R}_2$  is the amino acid residue of the  $\text{P}_2$  amino acid;

$\text{R}_3$  is the amino acid residue of the  $\text{P}_3$  amino acid;

$n$  is 0 or 1;

- 15  $m$  is 0 or 1; and

Het is a heterocyclic leaving group;

wherein the heterocyclic leaving group includes a four-, five-, six- or seven-membered ring having at least one C and at least one of N, O or S in the ring.

- 20 2. A cathepsin inhibitor according to claim 1 wherein  $n$  is 0 and  $m$  is 1.

3. A cathepsin inhibitor according to claim 2 wherein  $\text{R}_2$  is the residue of phenylalanyl (Phe).

4. A cathepsin inhibitor according to claim 3 wherein  
25  $\text{R}_1$  is a side chain such that the  $\text{P}_1$  amino acid residue is the residue of homophenylalanyl (HPhe).

5. A cathepsin inhibitor according to claim 4 wherein B is Mu.

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6. A cathepsin dipeptidyl peptidase inhibitor according to claim 1 wherein B = H.

7. A cathepsin inhibitor according to claim 5 wherein Het is a benzo-fused heterocycle.

5 8. A cathepsin inhibitor according to claim 5 wherein Het is dihydropyrone.

9. A cathepsin inhibitor according to claim 5 wherein Het is hydroxyproline or a salt, or extended peptide thereof.

10 10. A cathepsin inhibitor according to claim 5 where Het is a pyrone.

11. A cathepsin inhibitor according to claim 5 wherein Het is a pyridine.

12. A cathepsin inhibitor according to claim 5 wherein Het is pyrimidine.

15 13. A cathepsin inhibitor according to claim 5 wherein Het is a furan.

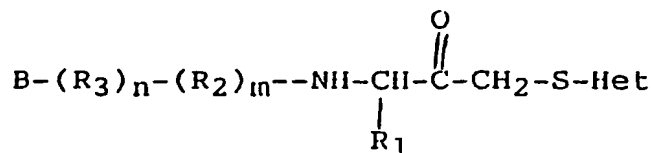
14. A cathepsin H inhibitor according to claim 6 wherein Het is a member of the group consisting of pyrone, pyridine, pyrimidine, furan or proline derivatives.

20 15. A cathepsin H inhibitor according to claim 7 wherein Het is a pyrone, pyridine, pyrimidine, furan or proline derivative and B = H, n = 0 and m = 0.

16. A composition according to claim 1, and further including a pharmaceutically acceptable carrier.

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17. Cathepsin inhibitors of the formula:



5 wherein

B is H or an amino acid blocking group for an N-terminal amino acid nitrogen;

R<sub>1</sub> is the amino acid side chain of the P<sub>1</sub> amino acid residue;

15 R<sub>2</sub> is the amino acid residue of the P<sub>2</sub> amino acid;

R<sub>3</sub> is the amino acid residue of the P<sub>2</sub> amino acid;

n is 0 or 1;

m is 0 or 1; and

Het is a heterocyclic leaving group;

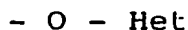
15 wherein the heterocyclic leaving group includes a four-, five-, six- or seven-membered ring having at least one C and at least one of N, O or S in the ring.

18. A cysteine protease inhibitor, comprising:

20 (a) a first portion which targets a desired cysteine protease and positions the inhibitor near the thiolate anion portion of the active site of the protease;

(b) a second portion which covalently bonds to the cysteine protease and irreversibly deactivates that protease by providing a carbonyl or carbonyl-equivalent which is  
25 attacked by the thiolate anion of the active site of the cysteine protease to sequentially cleave a heterocyclic leaving group;

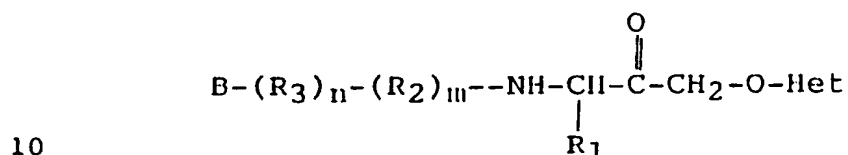
said heterocyclic leaving group being of the formula:



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wherein Het is a heterocycle having 4-7 members in the ring, with at least one of the heterocycle members being N, O or S.

19. A method of treating a patient for a medical condition, said method comprising administering to the patient a therapeutically effective amount of a composition of the formula:



wherein

B is H or an amino acid blocking group for an N-terminal amino acid nitrogen;

$\text{R}_1$  is the amino acid side chain of the  $\text{P}_1$  amino acid residue;

$\text{R}_2$  is the amino acid residue of the  $\text{P}_2$  amino acid;

$\text{R}_3$  is the amino acid residue of the  $\text{P}_2$  amino acid;

$n$  is 0 or 1;

$m$  is 0 or 1; and

Het is a heterocyclic leaving group;

wherein the heterocyclic leaving group includes a four-, five-, six- or seven-membered ring having at least one C and at least one of N, O or S in the ring.

20. The method of claim 19 wherein the medical condition is myocardial infarction.

21. The method of claim 19 wherein the medical condition is inflammation.

22. The method of claim 19 wherein the medical condition is a dystrophic disease.

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23. The method of claim 19 wherein the medical condition is rheumatoid arthritis.

24. The method of claim 19 wherein the medical condition is pneumocytis carinii.

5 25. The method of claim 19 wherein the medical condition is schistosomiasis.

26. The method of claim 19 wherein the medical condition is trypanosoma cruzi.

27. The method of claim 19 wherein the medical condition  
10 is trypanosoma brucei brucei.

28. The method of claim 19 wherein the medical condition is Crithidia fusiculata.

29. The method of claim 19 wherein the medical condition is malaria.

15 30. The method of claim 19 wherein the medical condition is periodontal disease.

31. The method of claim 19 wherein the medical condition is metachromatic leukodystrophy.

32. The method of claim 19 wherein the medical condition  
20 is muscular dystrophy.

33. The method of claim 19 wherein the medical condition is cancer metathesis.

34. The method of claim 19 wherein the medical condition is viral infection.

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35. The method of claim 19 wherein the medical condition is kidney disease.

36. The method of claim 19 wherein the medical condition is multiple sclerosis, peripheral nervous neuropathy or the  
5 like.

37. The method of claim 19 wherein the medical condition is polymyositis.

38. The method of claim 19 wherein the medical condition is a neurogenic disorder.

10 39. The method of claim 19 wherein the medical condition is Alzheimer's disease.

40. The method of claim 19 wherein the medical condition is leukemia.

15 41. The method of claim 19 wherein the medical condition is cataracts.

42. The method of claim 19 wherein the medical condition is an allergy.

43. The method of claim 19 wherein the medical condition is ischemia.

20 44. The method of claim 19 wherein the medical condition is bone resorption (osteo arthritis).

45. The method of claim 19 wherein the therapeutic composition includes a pharmaceutically acceptable carrier.

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## AMENDED CLAIMS

[received by the International Bureau on 1 May 1995 (01.05.95);  
original claim 16 amended; remaining claims unchanged (1 page)]

6. A cathepsin dipeptidyl peptidase inhibitor according to claim 1 wherein B = H.
7. A cathepsin inhibitor according to claim 5 wherein Het is a benzo-fused heterocycle.
- 5 8. A cathepsin inhibitor according to claim 5 wherein Het is dihydropyrone.
9. A cathepsin inhibitor according to claim 5 wherein Het is hydroxyproline or a salt, or extended peptide thereof.
- 10 10. A cathepsin inhibitor according to claim 5 where Het is a pyrone.
11. A cathepsin inhibitor according to claim 5 wherein Het is a pyridine.
12. A cathepsin inhibitor according to claim 5 wherein Het is pyrimidine.
- 15 13. A cathepsin inhibitor according to claim 5 wherein Het is a furan.
14. A cathepsin H inhibitor according to claim 6 wherein Het is a member of the group consisting of pyrone, pyridine, pyrimidine, furan or proline derivatives.
- 20 15. A cathepsin H inhibitor according to claim 7 wherein Het is a pyrone, pyridine, pyrimidine, furan or proline derivative and B = H, n = 0 and m = 0.
16. A cathepsin inhibitor according to claim 1, and further including a pharmaceutically acceptable carrier.

AMENDED SHEET (ARTICLE 19)

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/14061

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) :A61K 31/00, 37/00, 31/34; C07D 307/02; C07K 15/00.

US CL :549/479; 530/300, 331; 514/18,471.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 549/417; 514/460

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP, A, 163,162 (Okada et al) 27 June 1989.	1
A	WO, A, 93/09135 (Heng et al) 13 May 1993.	1

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	
*A* document defining the general state of the art which is not considered to be of particular relevance	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*E* earlier document published on or after the international filing date	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*O* document referring to an oral disclosure, use, exhibition or other means	*Z* document member of the same patent family
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

07 FEBRUARY 1995

Date of mailing of the international search report

01 MAR 1995

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**BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING**

This ISA found multiple inventions as follows:

- I. Claims 1-6,14,16,17,19,20,45(part of each) and 7, drawn to compounds and method of treating myocardial infarction, wherein Het is furan, classified in 549/479;514/471 .
- II. Claims 1-6,14,16,17,19,20,45(part of each) and 7, drawn to compounds and method of treating myocardial infarction, wherein Het is benzo-fused heterocycle, classified in various subclasses in classes 544,546 etc.
- III. Claims 1-6,14-16,17,19,20,45(part of each) and 9, drawn to compounds wherein Het is proline, classified in 548/532.
- IV. Claims 1-6,14-16,17,19,20,45(part of each) and 11, drawn to compounds wherein Het is pyridine, classified in 546/300.
- V. Claims 1-6,14-16,17,19,20,45(part of each) and 12, drawn to compounds wherein Het is pyrimidine, classified in 544/316.
- VI. Claims 1-6,14-16,17,19,20,45(part of each) and 13, drawn to compounds wherein Het is pyrone, classified in 549/417.
- VII. Claim 18, drawn to a cysteine protease inhibitor.
- VIII. Claims 19,45(part) and 21, drawn to method of treating inflammation.
- IX. Claims 19,45(part) and 22, drawn to method of treating dystrophic disease.
- X. Claims 19,45(part) and 23, drawn to method of treating rheumatoid arthritis.
- XI. Claims 19,45(part) and 24, drawn to method of treating pneumocytis carinii.
- XII. Claims 19,45(part) and 25, drawn to method of treating schistosomiasis.
- XIII. Claims 19,45(part),26and27, drawn to method of treating trypanosoma cruzi and/or trypanosoma brucei.
- XIV. Claims 19,45(part) and 28, drawn to method of treating crithidia fusciculata.
- XV. Claims 19,45(part) and 29, drawn to method of treating malaria.
- XVI. Claims 19,45(part) and 30, drawn to method of treating periodontal disease.
- XVII. Claims 19,45(part) and 31, drawn to method of treating metachromatic leukodystrophy.
- XVIII. Claims 19,45(part) and 32, drawn to method of treating muscular dystrophy.
- XIX. Claims 19,45(part) and 33, drawn to method of treating cancer metathesis.
- XX. Claims 19,45(part) and 34, drawn to method of treating viral infection.
- XXI. Claims 19,45(part) and 35, drawn to method of treating kidney disease.
- XXII. Claims 19,45(part) and 36, drawn to method of treating multiple sclerosis, peripheral nervous neuropathy.
- XXIII. Claims 19,45(part) and 37, drawn to method of treating polymyositis.
- XXIV. Claims 19,45(part) and 38, drawn to method of treating neurogenic disorder.
- XXV. Claims 19,45(part) and 39, drawn to method of treating Alzheimer's disease.
- XXVI. Claims 19,45(part) and 40, drawn to method of treating leukemia.
- XXVII. Claims 19,45(part) and 41, drawn to method of treating cataracts.
- XXVIII. Claims 19,45(part) and 42, drawn to method of treating allergy.
- XXIX. Claims 19,45(part) and 43, drawn to method of treating ischemia.
- XXX. Claims 19,45(part) and 44, drawn to method of treating bone resorption.

The inventions listed as Groups I-XXX do not meet the requirements for Unity of Invention for the following reasons: Compounds wherein Het is pyrone differs significantly from compounds wherein Het is benzo-fused heterocyclic ring system, proline, pyridine, pyrimidine or furan. They are differently classified. One of ordinary skill would not treat such diverse structures as functional equivalents of each other. The mere fact there is a single similarity, i.e. the presence of a furanyl moiety, is not in itself a significant reason to render the whole embodiment obvious. The various methods of use are independent and distinct. Art which anticipate or render obvious one group is not necessarily do the same for the remaining groups. Therefore, the above groups are not so linked as to form a single inventive concept. PCT Rule 13.3, 37 CFR 1.475(d).